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14. ABSTRACT

Metastatic breast cancer is a life threatening disease with limited treatment options. Molecular markers that can either predict accurately if a patient is likely to develop metastatic disease or be used as therapeutic targets are lacking. To address this, we have used a clinically relevant murine model of spontaneous metastasis to lung and bone, to identify candidate genes involved in metastatic progression. By microarray profiling and immunohistochemistry, we found enhanced expression of the cathepsin inhibitor Stefin A in epithelial cells isolated from primary tumors and their matched spontaneous metastases in lung and bone. In the human disease, patients lacking primary tumor expression of Stefin A had improved disease free survival and expression of Stefin A was enhanced in lung and bone metastases. As an endogenous inhibitor, we proposed that Stefin A is a marker of enhanced cathepsin activity in metastatic tumors. To explore this, we measured cathepsin activity using protease specific fluorescent substrates and found increased activity of cathepsin B in highly metastatic primary tumors. Stefin A and cathepsin B co-localise at the invasive fronts of primary tumors and in lung and bone metastases in the murine model and in human breast cancer. This study implicates cathepsins and their inhibitor Stefin A in breast cancer metastasis and provides evidence that Stefin A has prognostic value in breast cancer.

15. SUBJECT TERMS
Stefin A, breast cancer, metastasis, cathepsin

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Introduction

Breast cancer is treatable when detected at an early stage, yet is a serious clinical problem once it has progressed to metastatic disease in tissues such as lung and bone, where treatment is essentially palliative. Currently, it is not possible to predict with accuracy whether a patient is likely to develop distant metastases. This makes it difficult to tailor treatments specific to each patient and leads to over-treatment of those not likely to have recurrent disease. It is therefore crucial to determine mechanisms by which primary tumor cells migrate and develop metastases in secondary organs and to identify molecular targets for better therapeutic strategies to treat metastatic disease. To dissect the molecular pathogenesis of breast cancer, it is essential to use clinically relevant models that mimic the disease in humans.

Tumor progression and metastasis are regulated, in part, by the surrounding tissue microenvironment (1-3). The importance of tumor-stromal interactions in tumorigenesis and metastasis has highlighted the absolute requirement for *in vivo* models that allow for appropriate interactions. An ideal model is one that encompasses the entire process, including primary tumor formation and spontaneous metastasis to sites applicable to human disease. Unlike the commonly used xenograft models, the ideal animal model should be syngeneic to ensure tumor and host stroma compatibility and to allow the use of immunocompetent animals. Recent studies have demonstrated that matched host stroma is important in breast development and carcinogenesis (2, 4, 5) and the numerous roles of the immune system in tumorigenesis are well documented (6, 7).

A spontaneous metastasis model that mimics the clinical disease with primary tumor formation, invasion of cells through the stroma to the circulation and colonization at distant organs has been described (8). It is the only syngeneic model of the entire process and has great potential both for gene discovery and for analysis of the functional significance of gene candidates in breast cancer metastasis. In this model, sublines derived from a spontaneous mammary tumor in Balb/c mice are injected into the mammary gland and develop palpable tumors by day 10 and visible metastatic burden by 21-28 days. The model comprises sublines that are non-metastatic (67NR), weakly metastatic to lung (66cl4) or strongly metastatic to multiple organs including lung and bone (4T1.2, 4T1.13) (Figure 1) and permits analysis of changes in tumor cells and host stroma that promote distant metastasis (8). We have used this model to compare the gene expression profiles of purifed tumor cells derived from highly metastatic primary tumors (4T1.2, 4T1.13) to those from weakly and non-metastatic tumors (66cl4, 67NR). We have also isolated tumor cells from matched metastases in lung and bone and profiled changes associated with growth at the secondary site. The aim was to find novel molecular pathways involved in metastasis, but not in tumorigenesis. We wanted to identify gene candidates with altered expression in highly metastatic tumors, as potential prognostic factors, and also genes that have altered expression in associated metastases in lung and bone, as potential targets for therapy of metastatic disease.

One gene with enhanced expression with increasing tumor invasiveness was Stefin A. Stefin A is a physiological intracellular inhibitor of the cysteine family of cathepsin proteases (including cathepsin B, K, L, C, S) (9). Cathepsins are localized mainly in the lysosomes where they are involved in processes including apoptosis, antigen presentation, cellular homeostasis and autophagy (10). Additionally, secreted cathepsin K has a role in bone resorption and remodelling in osteoclasts (11). Recent studies have implicated the secretion of cysteine cathepsins during tumorigenesis and metastasis (12, 13). Secreted cathepsins (including L, B and K) promote tumor cell invasion through degradation of the extracellular matrix (ECM) and have prognostic significance in some cancers (14). The main objectives are to explore the expression and activity of Stefin A and the cysteine cathepsins during metastasis in the murine model and in human breast cancer and determine whether Stefin A is a potential prognostic indicator in breast cancer.

Body

Task 1: ***In situ hybridisation (ISH) of Stefin A1 to verify expression in the murine model***

Verification of Stefin A expression in the murine model of breast cancer has been completed. We have used a number of methods, including microarray, realtime RT-PCR, *in situ* hybridisation and immunohistochemistry to verify the enhanced expression of Stefin A in metastatic tumors.

Realtime RT-PCR of Stefin A expression

In our previous microarray analysis of alterations in tumour cells derived from metastatic primary tumors, we found that expression of the cathepsin inhibitor Stefin A1 was enhanced 7.6 fold in highly metastatic 4T1.2 and 4T1.13 primary tumor epithelium compared to tumor cells derived from non- or weakly-metastatic primary tumors (Table 1). This result was confirmed by realtime RT-PCR of RNA from immunopurified epithelial populations derived from the primary tumors (Figure 2A). Stefin A1 was expressed at levels significantly higher in immunopurified tumor cells from metastases when compared to the matched primary tumor, including lung metastases derived from the weakly metastatic 66cl4 tumor (Figure 2B). Expression was evident only in the context of the tumor microenvironment, with no expression in 4T1.2 and 4T1.13 cells in monoculture (data not shown). Whilst there is a single Stefin A homolog in humans, three homologs exist in the mouse (A1, A2 and A3). Since only Stefin A1 was present on the custom 15K array, we used primers that distinguish between Stefin A1, A2 and A3 and real time RT-PCR, to confirm that all homologs are induced in highly metastatic primary tumors (Figure 2C).*:*

Description	Common	Fold Difference	P value	Function
stefin A1	stfa1	↑ 7.6	0.007	cystatin, cathepsin inhibitor
breast cancer metastasis-suppressor 1	Brms1	2.2 ↓	0.002	Supression of metastasis
ets variant gene 6 (TEL oncogene)	ETV6	↑ 7.2	0.004	cellular aggregation, transformation
complement component 3	C3	↑ 3.8	0.004	up in cancer patients, including highly metastatic human melanoma cells
histidine decarboxylase	Hdc	↑ 3.5	0.003	synthesis of histamine, tumor cell proliferation
SMT3 (supressor of mif two, 3) homolog 1	Smt3h1	↑ 3.9	0.001	protection against TNF etc cell death, cell cycle progression
histidine decarboxylase	Hdc	↑ 3.5	0.003	synthesis of histamine, tumor cell proliferation
dachshund 1 (Drosophila)	DACH1	↑ 2.4	0.004	cell proliferation, inhibits apoptosis (TGFB), developmental gene
bone morphogenetic protein 4	BMP4	2.5 ↓	6.00E-05	member of TGF-beta superfamily, induce cell senescence
topoisomerase (DNA) I	Top1	2.1 ↓	0.001	relaxation of supercolied DNA through breakage, cell cycle checkpoint
mitogen activated protein kinase 1	MAPK1	2.4 ↓	0.005	activation of ERK1/ERK2, roles in differentiation
MAP kinase-interacting serine/threonine kinase 2	Mknk2	2.6 ↓	0.003	limit/inhibit translation
developmental pluripotency associated 5	dppa5	↑ 3	7.00E-04	development
maternal effect gene	Mater	↑ 2.2	0.004	early development
eomesodermin homolog (Xenopus laevis)	Eomes	↑ 1.8	0.006	early developmental gene
envoplakin	Evpl	↑ 2.6	0.008	cytolinker protein
Rho GTPase activating protein 8	Arhgap8	↑ 2.3	0.004	cytoskeletal organization, cell cycle regulation, ras-mediated transformation
Rho guanine nucleotide exchange factor (GEF) 3	Arhgef3	↑ 1.8	0.008	cytoskeletal rearrangement
ARP3 actin-related protein 3 homolog (yeast)	ARP3	2.6 ↓	0.006	actin nucleation, organization of the cytoskeleton, controls polarised cell growth
heat shock protein 8	HSP8/73	3 ↓	0.01	chaperone
autophagy 7-like (S. cerevisiae)	Apg7l	3 ↓	0.002	enzyme essential for autophagy
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	3 ↓	0.003	stress induced, induced upon anti-metastatic activity (lung adenocarcinoma cells)
zinc finger protein X-linked	Zfx	3.1 ↓	0.004	inhibits angiogenesis, involved in embryonic growth (sex differentiation)

Table 1- Genes altered in highly metastatic epithelial cells. Microarray profiling revealed genes that had increased (↑) or decreased (↓) expression in immunopurifed epithelial cells from primary tumors that metastasize to lung and bone when compared to weakly or non-metastatic tumors.

Affymetrix Microarray Expression Profiling

Tumor cells from primary tumors and metastases were purified with anti-mouse Ep-CAM (BD Biosciences Pharmingen, San Diego, CA) pre-conjugated to sheep anti-rat Dynabeads (Dynal Biotech). Matched pairs of isolated tumor cells from four 4T1.2 primary tumors and spine metastases were used for microarray analysis. The Affymetrix Two-Cycle Amplification Kit was used to amplify the RNA and amplified RNA (aRNA) was hybridized to Mouse Genome 430 v2.0 microarrays (Affymetrix, Santa Clara, CA). Array data was analyzed using Genespring GX 7.3 (Agilent Technologies). Stefin A1 and A3 (but not A2) oligonucleotides are present on the mouse genome 430 v2.0 Affymetrix array. When tumor

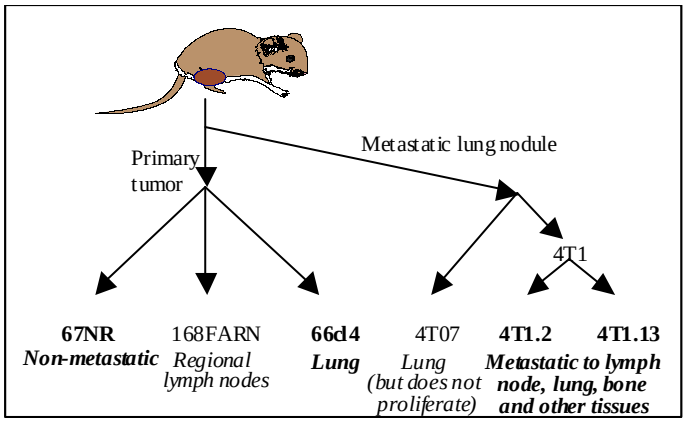


Figure 1. Orthotopic model of breast cancer metastasis to bone. Several tumor sublines have been isolated from a spontaneously arising mammary gland carcinoma. Each subline has a distinct metastatic phenotype. 67NR is non-metastatic, while 168FARN, 66cl4 and 4T07 are weakly metastatic. 4T1.2 and 4T1.13 are two bone metastasizing tumor clones derived from the lung metastasizing 4T1 subline.

cells from spine metastases were normalized to their matched primary tumor cells , enhanced expression of both Stefin A1 and A3 was found in all four biological replicates, as illustrated in the heat map (Figure 2D). In fact, both Stefin A1 and A3 were expressed at least 20-fold higher in the matched metastases ($p<0.03$). This confirms the up-regulation of Stefin A1 in 4T1.13 spine metastases compared to matched primaries that was observed using the custom cDNA array (data not shown), and the enhanced expression of Stefin A seen in metastatic lesions by realtime RT-PCR in Figure 2B.

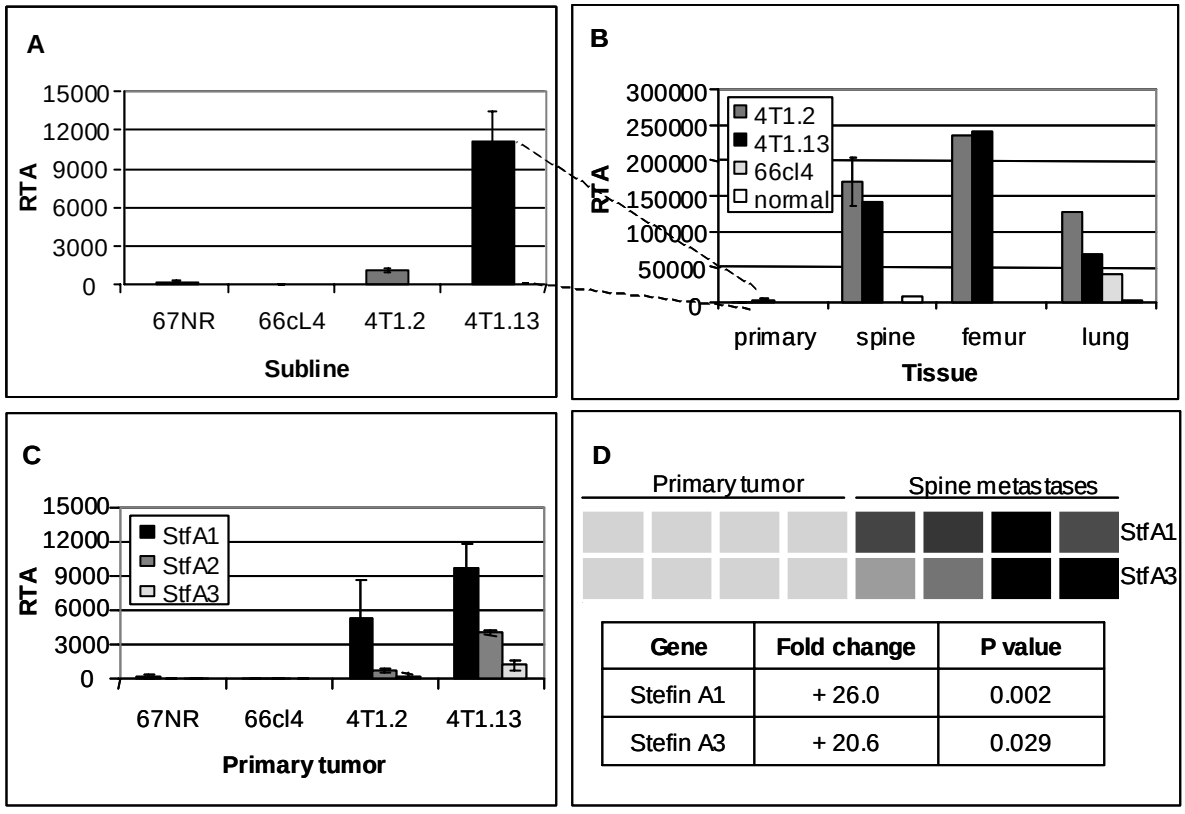


Figure 2 Stefin A transcript expression in primary tumors and metastases
RNA isolated from tumor cells derived from primary tumors (A) and matched metastases (B) were reverse transcribed and real-time RT-PCR was used to detect Stefin A1 expression relative to GAPDH. C) Expression of the three murine Stefin A homologs in RNA extracted from whole primary tumors. D) Microarray comparison of Stefin A1 and A3 expression in four replicate samples of tumor cells purified from primary and matched spine metastases. Signal intensity in spine metastases was normalized to matched primary samples.

In situ Hybridisation

Using *in situ* hybridisation, the increased expression of Stefin A1 throughout metastatic progression was verified histologically. Riboprobes were generated by end-labelling 5' and 3' ends of PCR primers with T7 and SP6 promoter sequences and PCR amplification of DNA sequences of interest followed by *in vitro* transcription using T7 and SP6 polymerase, generating sense and anti-sense probes respectively. The *in vitro* transcription included labelling of transcripts with FITC. Paraffin embedded sections of 67NR, 66cl4, 4T1.2 and 4T1.13 primary mammary tumors and their corresponding metastases were used for *in situ* hybridisation (ISH). Protocols were modified from those previously described (15, 16), including deparaffinization and fixation of tissues, pre-treatment for access to target nucleic acid sequence and riboprobe hybridization overnight, with the use of FITC riboprobe labelling replacing DIG as previously used. Riboprobe/FITC signal was detected and amplified using the GenPoint™ Fluorescein Tyramide Signal Amplification System (DakoCytomation). As a positive control, mouse embryos were stained to reveal positive cells within the liver at day 15.5. By staining sections from primary mammary tumors, expression (positive staining) was observed in only the highly metastatic 4T1.2 and 4T1.13 tumors, and such expression was limited to only specific subsets of tumor cells (Figure 3). In contrast, sections from matched lung metastases revealed staining of metastatic lesions that appeared in a large proportion of tumor cells (Figure 3). This supports the hypothesis that the higher levels of expression observed in metastatic lesions compared to primary tumors detected by quantitative RT-PCR was due to only a subset of cells in the primary tumor expressing the gene and either a selection of these cells that metastasise and grow in distant sites, or induction of stefin A1 in tumor cells once they reached the microenvironment of the lung or bone. As Stefin A is only a small gene (290 bp), only one probe could be used for ISH detection and we usually use at least 2 probes that span the gene, each being ~500 bases long. This resulted in staining that was less intense than is preferable. Chemicon now has an anti-mouse Stefin A antibody that we used for immunohistochemistry.

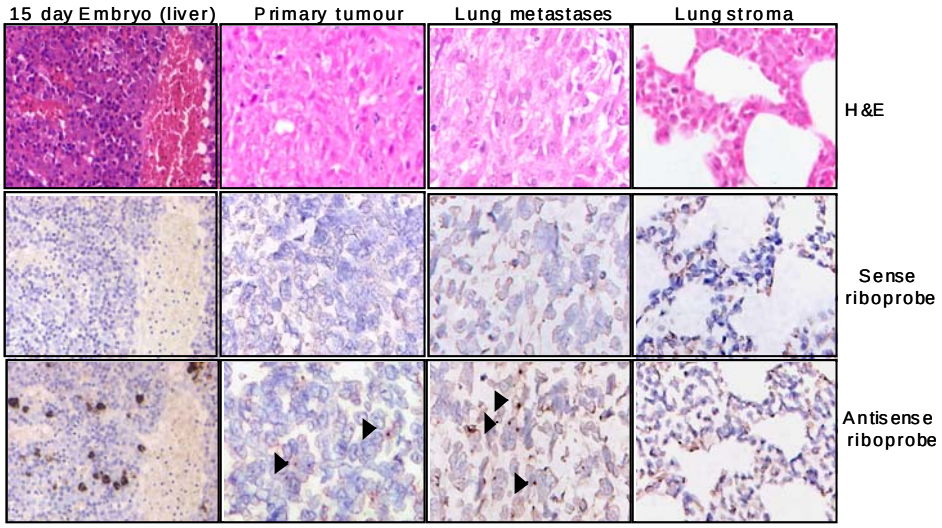


Figure 3 Expression of Stefin A1 in primary and metastatic tumors. Expression of Stefin A1 was validated by *in situ* hybridisation. 4T1.2 primary tumors and lung metastases were stained with stefin A1 antisense riboprobes and sense probes as controls. Riboprobes were FITC labelled and detected using anti-FITC HRP followed by DAB staining and counter staining with hematoxylin. Tumor staining (brown) is indicated by arrows. Mouse embryo (15.5 days) was stained as a positive control and staining was detected in the liver.

Immunohistochemistry

To verify protein expression, normal mammary gland and 67NR, 66cl4, 4T1.2 and 4T1.13 primary tumors were analysed for presence of Stefin A using an antibody targetting the N-terminal region that recognizes

all three homologs. By immunohistochemistry, we detected Stefin A only in the metastatic 4T1.2 and 4T1.13 primary tumors (Figure 4). Expression was higher at the periphery of the tumor, in contact with surrounding stroma. This was also the case in matched lung and bone metastases, where Stefin A was localized predominantly in tumor cells interacting with stroma (Figure 4). In tumor-bearing spines, but not in normal spine, scattered inflammatory cells in regions adjacent to the tumor were positive for Stefin A (Figure 4).

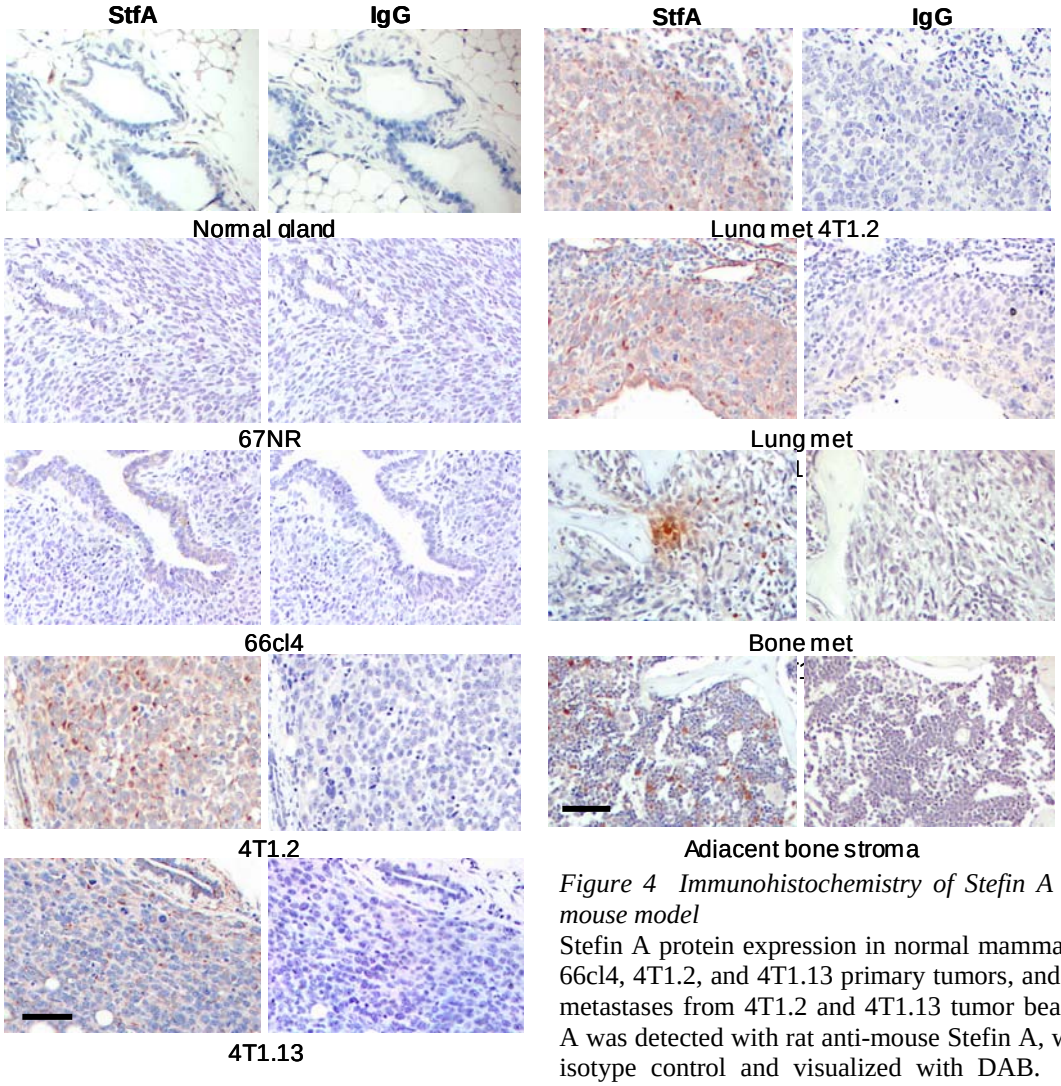


Figure 4 Immunohistochemistry of Stefin A expression in the mouse model

Stefin A protein expression in normal mammary fat pad, 67NR, 66cl4, 4T1.2, and 4T1.13 primary tumors, and in lung and spine metastases from 4T1.2 and 4T1.13 tumor bearing mice. Stefin A was detected with rat anti-mouse Stefin A, with rat IgG_2 as an isotype control and visualized with DAB. All tissues were counterstained with hematoxylin. Scale bar represents 50 µm.

Task 2: ***Verify the expression of Stefin A in human breast cancer***

To determine the clinical relevance of Stefin A as a marker of metastasis, we analysed expression in human tissues. In normal reduction mammoplasty tissue, Stefin A was frequently expressed in the myoepithelial layer surrounding normal ducts and lobules (Figure 5A). The luminal epithelium, from which the majority breast tumors arise, was negative in numerous normal breast tissue samples from different individuals. In contrast, a subset of primary tumors expressed Stefin A (Figure 5B, C). Since the murine studies revealed increased Stefin A expression in lung and bone metastases, human lung and bone metastases were also analysed. As in our murine model, tumor deposits in lung (Figure 5D) and bone (Figure 5E, F) expressed high levels of Stefin A. Again, the strongest staining was in tumor deposits that

were interacting with the lung or bone stroma. In regions of lung metastases where cells were not in contact with stromal cells, Stefin A was not expressed (data not shown).

A small cohort of primary tumors (n=25) from patients with known outcome was used to investigate the prognostic significance of Stefin A. Primary tumors that formed metastases in lung or bone expressed Stefin A with much higher frequency than non-metastatic primary tumors. This was indicated by univariate disease-free survival (DFS) analysis that showed a significant correlation between improved outcome and lack of Stefin A tumor expression (p=0.017)(Figure 5G). Although a trend was observed between Stefin A expression and risk of cancer-related death, this relationship was not significant (p=0.087) due to the small sample size (Figure 5H). We have also stained and scored a large cohort (>200

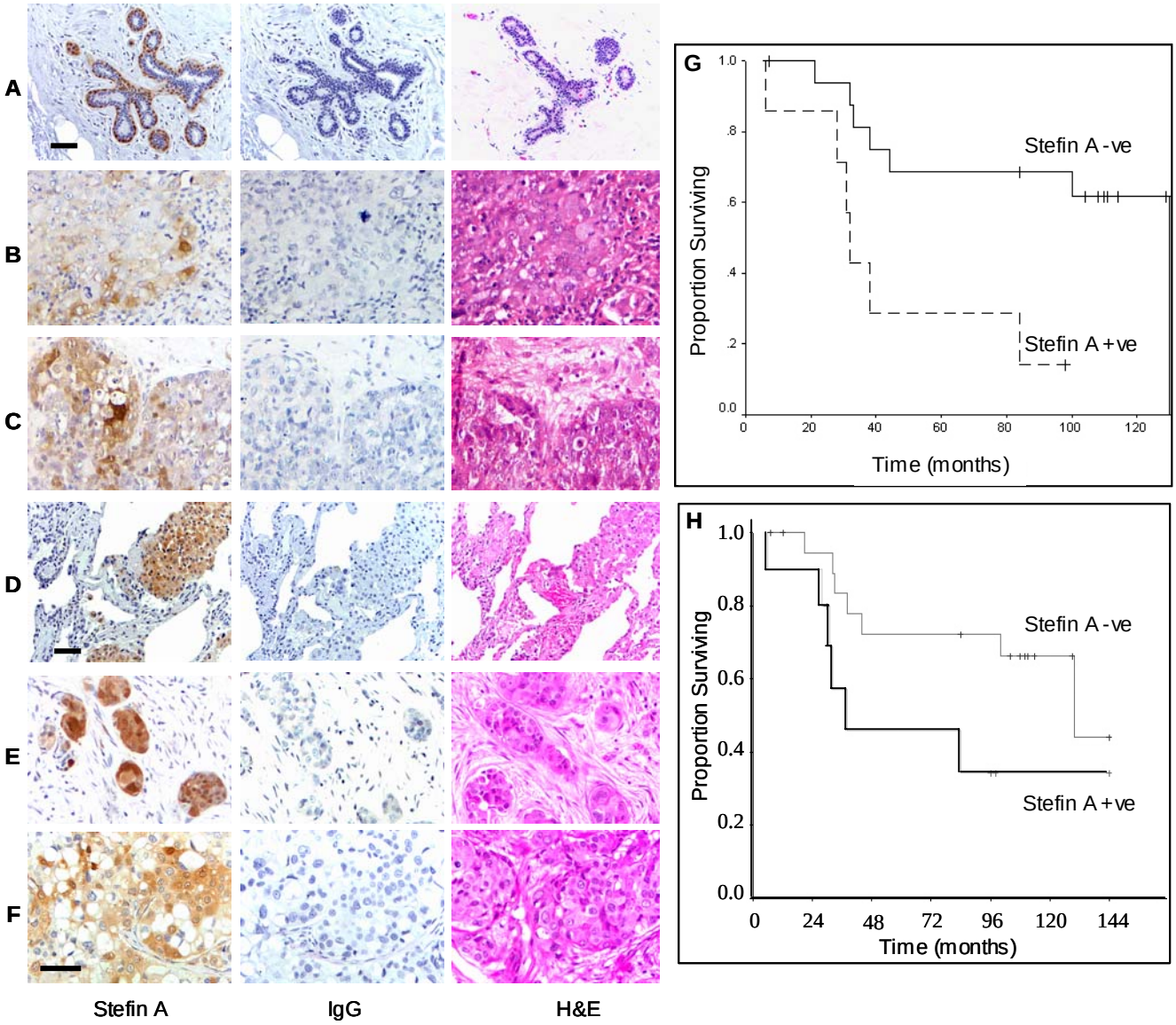
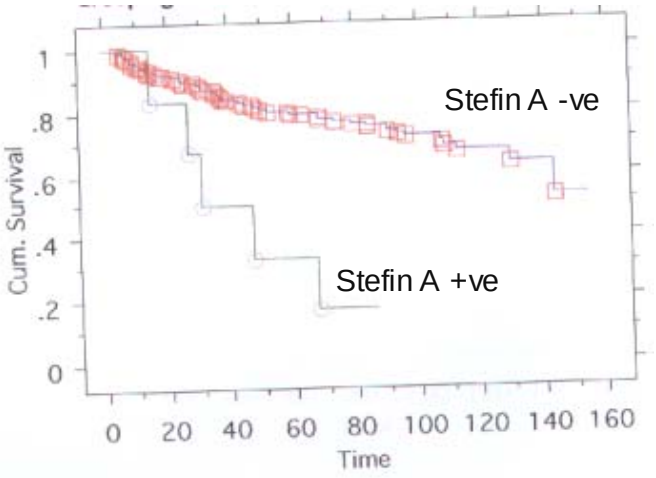


Figure 5 Immunohistochemistry of Stefin A expression in human tissues

Sections of formalin fixed, paraffin embedded reduction mammoplasty tissue (A), primary breast tumors (B,C), or metastases in lung (D) and bone (E,F) were stained with mouse anti-human Stefin A or 1B5 hybridoma supernatant control and visualized with DAB. All tissues were counterstained with hematoxylin. Scale bar represents 50 µm. Kaplan-Meier survival curve comparing disease free survival (G) or death due to disease (H) in patients expressing Stefin A and patients lacking Stefin A expression in the primary tumor. Stefin A expression was measured in primary breast tumor tissues from a cohort of 25 patients with known outcome.

patient samples) of primary breast tumors from the Garvan Insititute, Sydney and we are waiting for final analysis. Preliminary analysis, however, has shown that Stefin A expression does correlate with increased risk of metastatic recurrence (Logrank (Mantel-Cox) test, p= 0.0002) (Figure 6), yet this only represents a subpopulation of Stefin A positive tumors and will be updated upon completion of analysis.

Figure 6: Stefin A expression correlates with risk of metastatic recurrence in a patient cohort
Kaplan-Meier cumulative survival curve comparing disease free survival in patients expressing Stefin A and patients lacking Stefin A expression in the primary tumor. Stefin A expression was measured in primary breast tumor tissues from a cohort of >200 patients with known outcome.



Task 3*: Investigate the functional role of Stefin A in vitro*
Due to the impact of the microenvironment on Stefin A expression, we decided to investigate the induction of Stefin A in co-cultures *in vitro*. Expression is evident only in the context of the tumor microenvironment, with no expression in 4T1.2 and 4T1.13 cells in monoculture (data not shown). We wanted to determine whether we could stimulate the expression of Stefin A in these cells when co-cultured with stromal cells *in vitro* to investigate the effect of the surrounding microenvironment. We were able to induce Stefin A to modest levels in co-cultures of 4T1.2 cells with mammary gland stromal cells (Figure 7) and also with primary mammary fibroblasts and ECM components, however expression was lower than that observed *in vivo* and may indicate additional factors that are required to induce Stefin A.

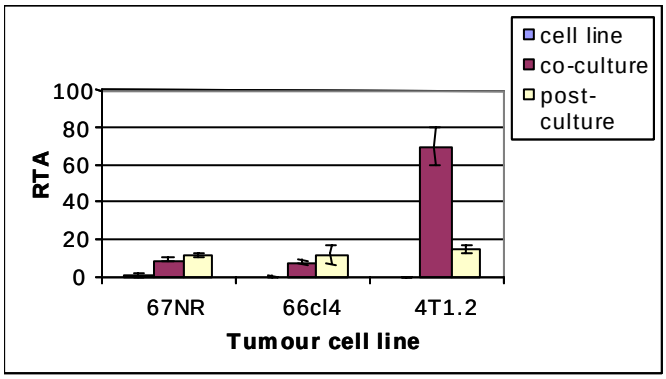


Figure 7 Co-culture induction of Stefin A1
Mammary fat pads were resected and collagenase A digested into single cell suspensions. Filtered cells were then plated and allowed to attach overnight. For contact co-cultures tumor cells (67NR, 66cl4 or 4T1.2) were then added to stromal populations (co) or cultured separately (cell line) overnight in serum-free media. Post-culture represents cells that were incubated separately and then mixed in lysis buffer to determine the additive expression of Stefin A1 in tumor and stromal cells when not cultured together. Expression was detected using quantitative RT-PCR of Stefin A1 and a comparison to GAPDH.

It remains to be investigated whether there are changes in the activity of specific cathepsins, that may subsequently lead to Stefin A1 expression as a mechanism of inhibition. We are now investigating the possible mode of Stefin A1 induction, including the role of cathepsins (since Stefin A1 is an intracellular inhibitor of cathepsins) and whether stromal cells isolated from other organs (tissues that have metastatic growth in the 4T1.2 model and those that do not develop any tumor burden after primary tumor formation) can also induce Stefin A1 expression upon contact co-culture. Due to the difficulty we have faced so far inducing Stefin A expression in cells in

culture to levels in the range of that seen in culture, we may need to functionally dissect the role of cathepsins and their inhibitor Stefin A using *in vivo* studies.

Task 4: ***Evaluate the effect of Stefin A1 expression on primary tumor growth and spontaneous metastasis to lung and bone in vivo***.

It was of interest to determine whether enhancing the expression of Stefin A1 in 4T1.2neo1 cells *in vitro* would alter the metastatic burden when cells were injected into the mammary fat pad. Stefin A1 was cloned into the pBabe vector (containing a puromycin resistance gene) and transfected into the phoenix viral packaging line. The 4T1.2 cells were then infected with virus either containing the pBabe base vector (BV, as a control) or the pBabe-Stefin A1 plasmid and stably selected by growth in puromycin. Expression was confirmed by quantitative RT-PCR (Figure 8) and high expressers were pooled for *in vivo* studies.

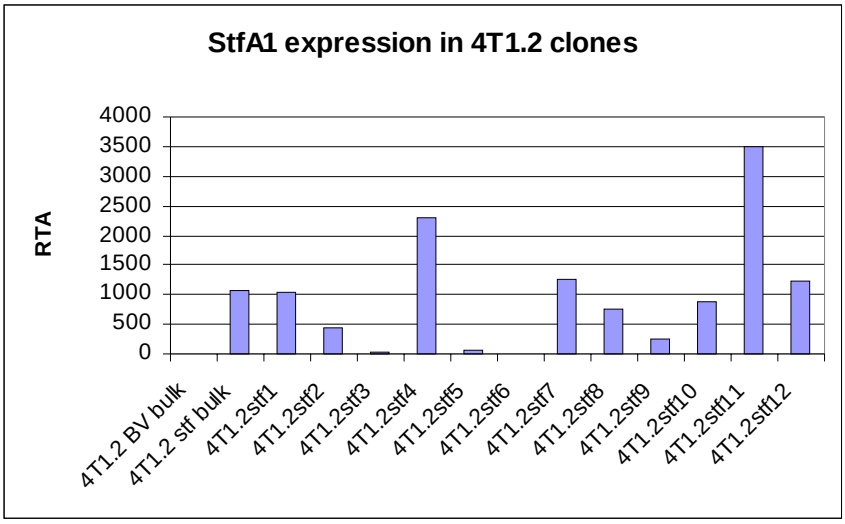
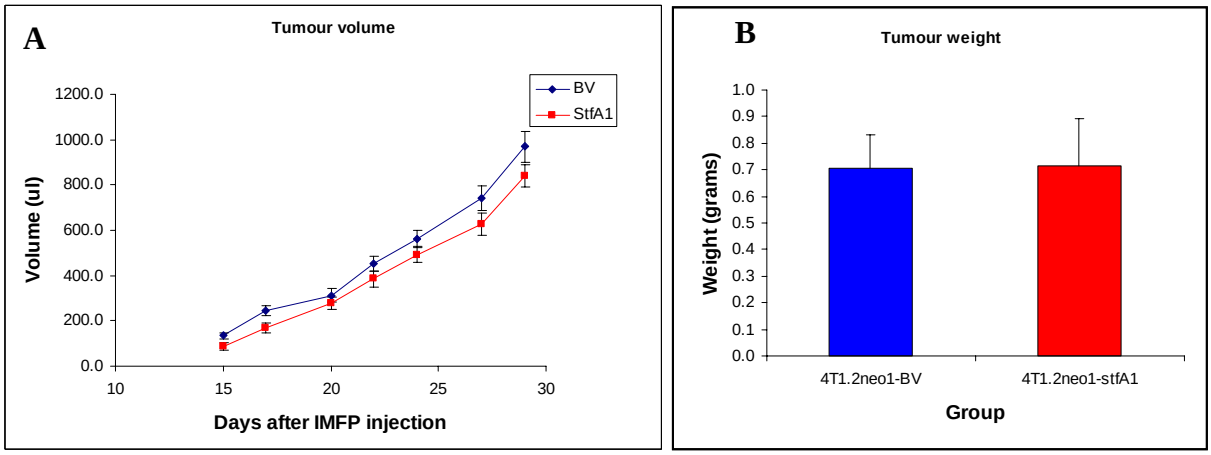


Figure 8 Stefin A1 over-expression in 4T1.2 cells does not effect primary tumor growth in vivo

Bulk 4T1.2 neo1-StfA1 cells were single cell cloned and quantitative RT-PCR was used to detect Stefin A1expression. High expressers were pooled (clones 1, 4, 7, 11, 12) and used for *in vivo* experiments along with the 4T1.2neo1-base vector (BV) bulk cell population as a control.



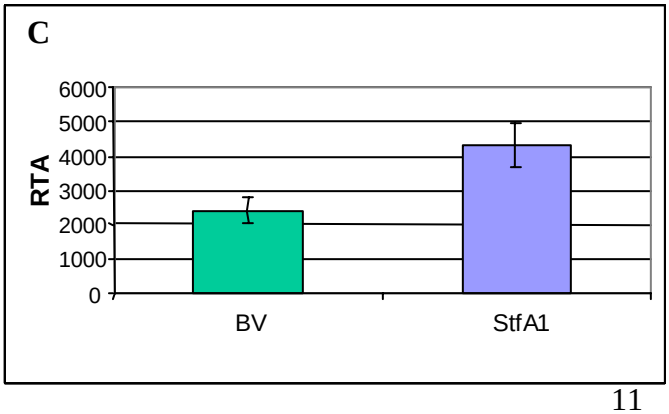


Figure 9 Stefin A1 over-expression in 4T1.2 cells does not effect primary tumor growth in vivo

High Stefin A expressers were pooled and used for *in vivo* experiments along with the 4T1.2neo1-base vector (BV) bulk cell population as a control. Primary tumor growth and weight at time of harvest was not significantly different between the 2 groups (B, C), indicating that Stefin A1 expression does not alter the growth of the primary tumor. Primary tumors derived from injection of 4T1.2neo1-BV cells gained expression of Stefin A1 (as expected by previous *in vivo* studies) yet the 4T1.2-StfA1 clones maintained an average 2-fold higher expression (C)

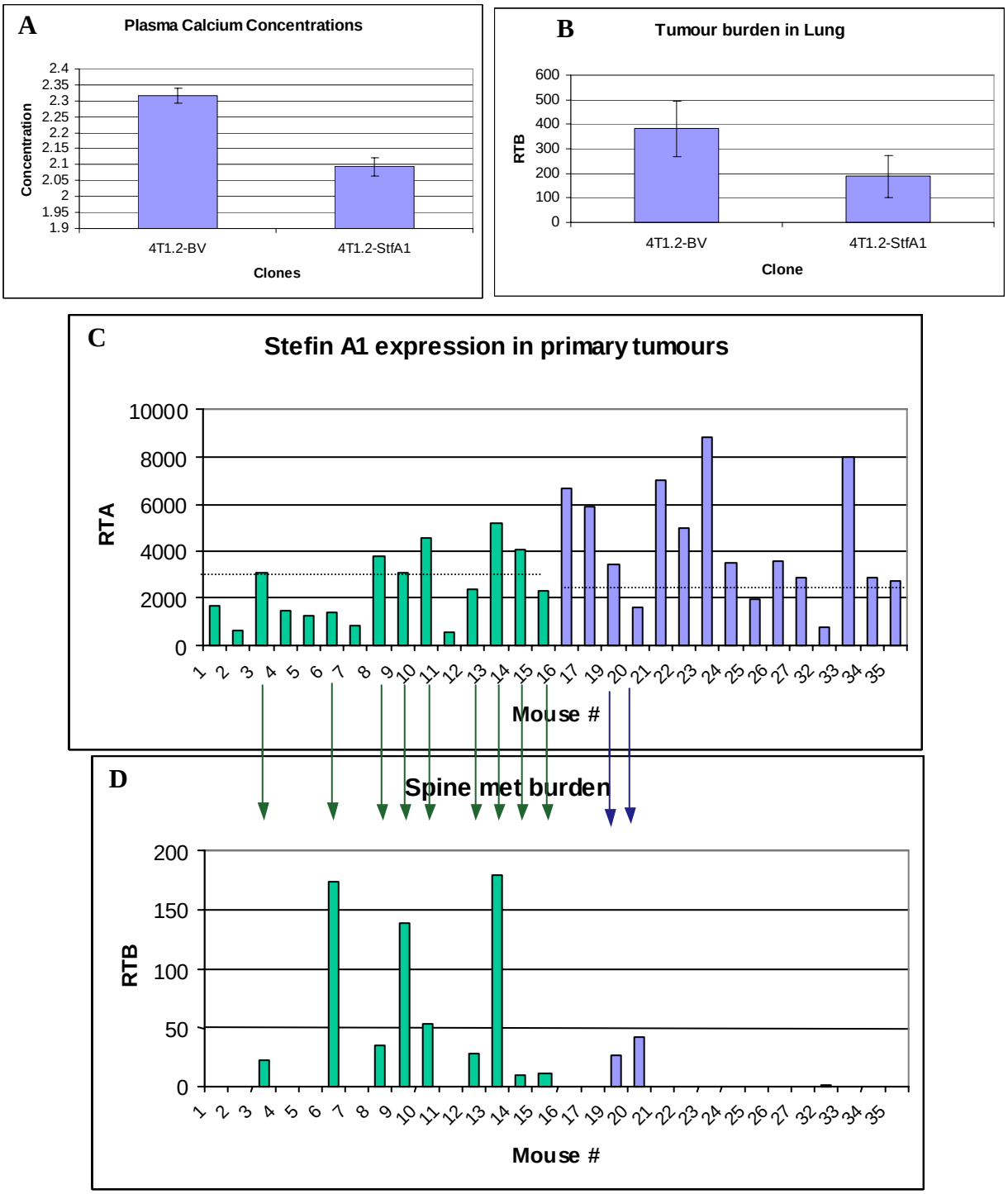


Figure 10 Stefin A1 over-expression in vitro leads to reduced lung and bone metastasis in vivo

A) The concentration of plasma calcium in all 30 mice was measured as an indication of distant metastatic involvement. As can be seen, mice injected with 4T1.2-StfA1 clones had reduced plasma calcium, in fact the concentration was equivalent to levels seen in mice that do not have tumor burden (~2.1). This indicates a decrease in metastatic burden and this was confirmed by realtime QPCR detection. In the lungs (B) there was a marked decrease in tumor burden (neomycin tagged tumor cells compared to vimentin signal). This effect was even greater in spine metastases, with an almost complete inhibition of spine metastases in the 4T1.2 cells over-expressing StfA1 (mice 16-35, blue)(D). When spine metastatic burden is compared to Stefin A1 expression in the primary tumor of each mouse (C), the highest stefin A1 expression correlated with a lack of metastasis.

When Stefin A1 and BV clones were injected into the mammary glands of Balb/c mice there was no significant difference in primary tumor growth (Figure 9A,B), and even though Stefin A1 was induced in the 4T1.2-BV primary tumors (as expected), the enhanced expression was maintained in 4T1.2-StfA1 tumors (Figure 9C). Interestingly, both plasma calcium levels (Figure 10A) and lung and bone metastases (Figure 10B,D) decreased in the over-expression lines. This indicated that over-expression of Stefin A1 decreases metastasis, in fact it almost totally inhibits bone metastasis with most spines having no detection of any tumor burden (by QPCR of the neomycin tag) and the return of plasma calcium concentrations to that of "normal" mice that do not have tumor burden. The expression of Stefin A1 in primary tumors and the spine metastatic burden was compared to reveal whether there is a correlation between Stefin A1 expression and metastasis. In 4T1.2-BV cells, moderate Stefin A1 levels correlated with increased metastasis (as seen by previous verification studies) whereas in the 4T1.2-StfA1 clones, primary tumors with much higher levels of stefin A1 do not metastasise at all, which is also the case when there is very low levels of the gene expressed in the primary tumor. This needs to be investigated further, but it seems that there is a threshold of Stefin A1 expression that correlates with metastasis and expression levels considerably above or below this level do not have spine metastatic involvement. There are a number of hypotheses as to why increased Stefin A1 may decrease metastasis. Firstly, it was seen from task 2 that Stefin A1 is only seen in specific cell populations within a primary tumor. Expression in all cells prior to injection may inhibit cathepsins that are important in metastasis (eg cathepsin B, D, K). Alternatively, the cellular localization of stefin A1 may be altered, again resulting in the inhibition of cathepsins that are pro-metastatic. In base vector cells, the localised expression of Stefin A1 may be serving to inhibit the lysosomal activity of pro-apoptotic cathepsins (eg cathepsin L) and those involved in immune recognition/antigen presentation (eg cathepsin S). Another hypothesis is that Stefin A1 induction in 4T1.2 cells *in vivo* may just be a marker of an increased cathepsin activity. We are currently investigating cathepsin expression and activity in the mouse model to determine whether Stefin A co-localises with cathepsins in vivo and the specific cathepsins that have a role in metastasis.

Cathepsin expression and activity

Due to the role of Stefin A as a cathepsin inhibitor it is important to determine if the enhanced expression in metastatic nodules is in response to increased cysteine cathepsin activity. We propose that Stefin A is acting as a protease sensing system and hence, a marker of the contribution of cathepsins to metastasis.

We quantitated the expression of cathepsins B, L, K and S in 67NR, 66cl4 and 4T1.2 primary tumours and found that transcript levels did not correlate with metastatic potential of the primary tumours (Figure 11A). However, when fluorogenic substrates were used to assay cathepsin activity in the same tissues, a significant increase in cathepsin B activity was found in the highly metastatic primary tumours compared to non-metastatic 67NR tumours ($p=0.008$) (Figure 11B). Conversely, cathepsin L activity decreased with increasing metastatic potential ($p= 0.006$) (Figure 11C) whilst cathepsin S activity was unchanged (Figure 11D). Immunohistochemical analysis in 4T1.2 primary tumours revealed strong cathepsin B protein expression at the invasive front but no expression was detected in areas not adjacent to surrounding stroma (Figure 12). Cathepsin B was expressed in matched spine metastases, with greatest intensity in tumour cells adjacent to bone and other stromal components (Figure 12). This pattern of expression was similar to that seen with Stefin A, where expression is greatest in tumour cells interacting with host stroma (Figure 2).

To establish a functional connection between cathepsin B and Stefin A in primary and metastatic human tumours, we compared their expression by IHC and found co-localisation of Stefin A with cathepsin B in multiple invasive primary tumours (Figure 13A). This is consistent with Stefin A acting as a biomarker of elevated cathepsin B expression. Additionally, in lung metastases, where Stefin A is commonly expressed in tumour deposits interacting with stroma, cathepsin B was specifically expressed in the same deposits, as identified using serial sections (Figure 13B). In regions that were not interacting directly with the ECM or stroma, neither Stefin A nor cathepsin B expression was detectable (Figure 13C). Additionally, in lung metastases, where Stefin A is commonly expressed in tumor deposits interacting with stroma, cathepsin B

was specifically expressed in the same deposits, as identified using serial sections (Figure 14A). In regions that were not interacting directly with the ECM or stroma, neither Stefin A nor cathepsin B expression was detectable (Figure 14B). This co-localization was also observed in metastatic nodules in the murine model (data not shown).

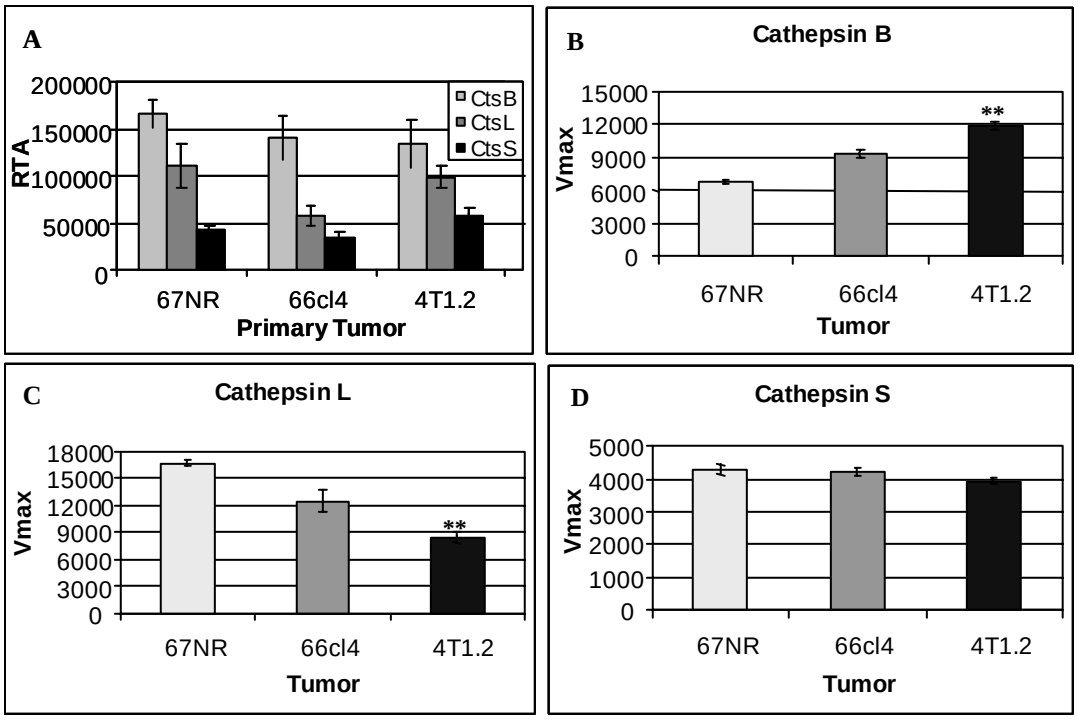


Figure 11 Cathepsin expression and activity in primary tumors
Snap frozen tissues (67NR, 66cl4, 4T1.2 primary tumors) were pulverized to a powder form in liquid nitrogen. To quantitate cathepsin transcripts, RNA was extracted and real time RT-PCR was used to determine cathepsin B (CtsB), L (CtsL) and S (CtsS) expression compared to GAPDH (A). For cathepsin activity assays, cells were lysed in the appropriate buffer (BioVision) and protein concentrations determined by Bradford assay. Lysates containing 50 mg protein were added to cathepsin B, L, and S activity assays utilizing fluorogenic substrates for detection of activity (BioVision) (B-D). ** Indicates P values of <0.01 between 67NR and 4T1.2 primary tumors.

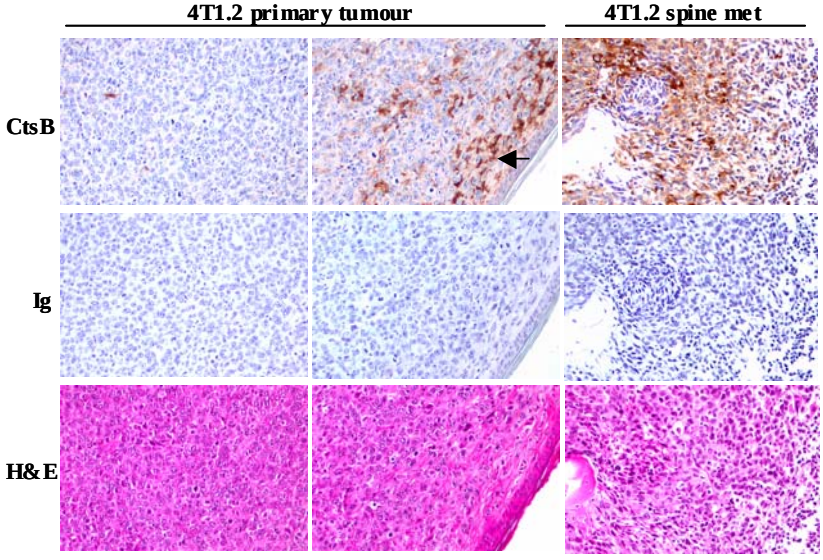


Figure 12 Cathepsin B expression in 4T1.2 primary tumours and spine metastases. Paraffin embedded primary and spine tissues were stained with rabbit anti-mouse cathepsin B (CtsB) or rabbit Ig isotype control. Two panels designated primary tumour represent two regions of the same tumour, either in the centre (left panel) or at the invasive front (indicated by the arrow) (centre panel).

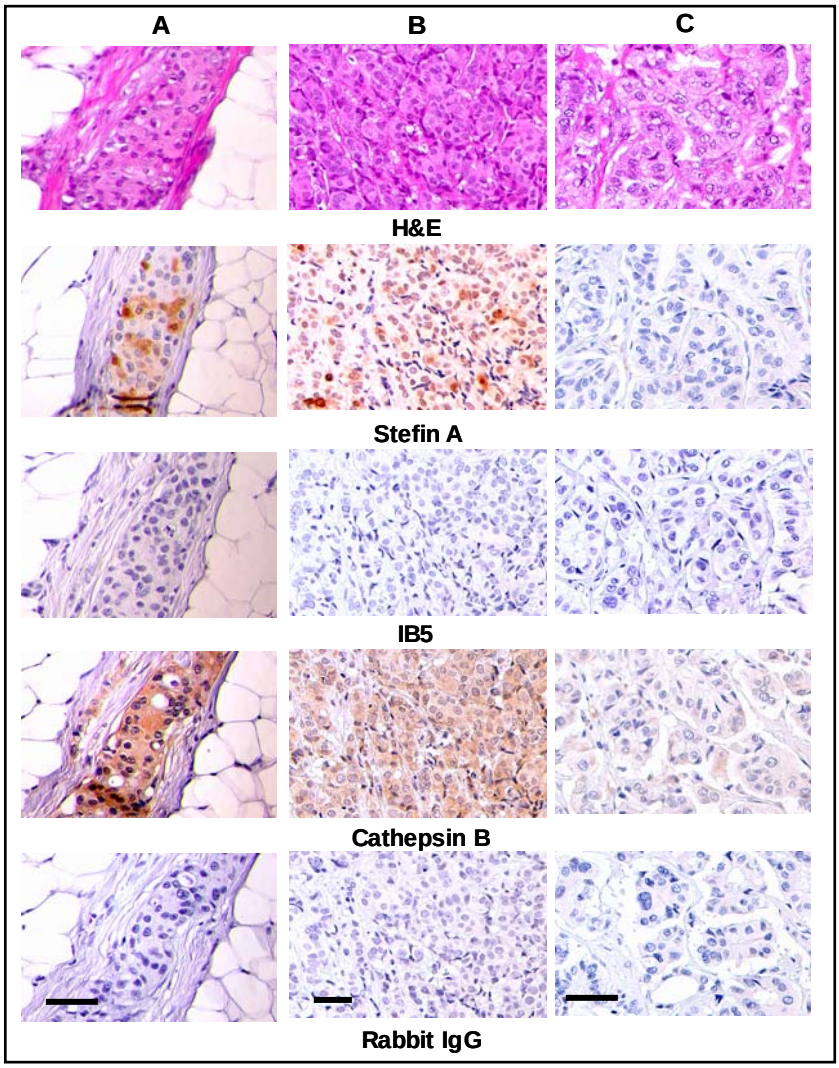


Figure 13 Cathepsin B expression in human primary tumors
Sections of formalin fixed, paraffin embedded primary breast tumors (A-C) were stained with Stefin A monoclonal antibody and 1B5 hybridoma supernatant as isotype control, or polyclonal anti-cathepsin B and rabbit IgG as a negative control. Signal was visualised with DAB and all tissues were counterstained with hematoxylin. Sample D exhibits no Stefin A protein and very little cathepsin B. Scale bar represents 50 μm.

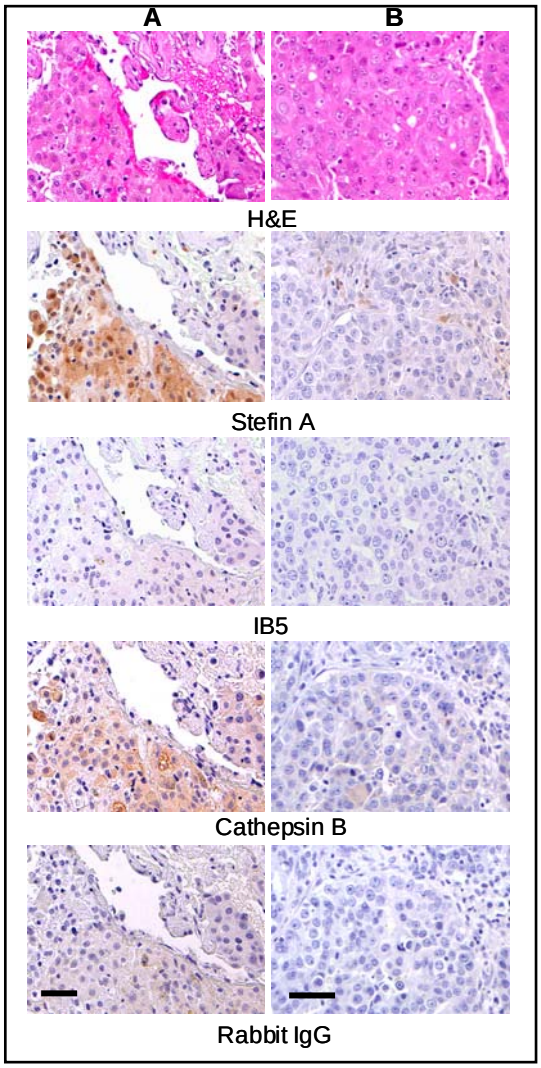


Figure 14 Cathepsin B co-localises with Stefin A in human lung metastases
Correlation of Stefin A and cathepsin B expression in paraffin embedded lung metastases using immunohistochemistry. Tumor deposits in contact with lung stroma express Stefin A (A) whereas the centre of the tumor is negative (B). Scale bar represents 50 μm.

Using this concept award, we have verified that Stefin A is a marker of metastasis in breast cancer. Our future efforts will include determination of whether key cathepsin proteases and causing the induction of Stefin A and if inhibiting such proteases has therapeutic potential for treatment of metastatic breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments during the course of this Concept Award are as follows-

- Verification that expression of the cathepsin inhibitor Stefin A (including the 3 murine homologs A1, A2 and A3) is induced in primary tumours that are metastatic to lung and bone, and is expressed at greater levels in matched metastases in a murine model of breast cancer metastasis.

- In human cancer, Stefin A expression is also enhanced throughout metastasis, with highest expression observed in metastases in lung and bone. Staining of a small cohort of primary breast cancers indicates that Stefin A expression predicts poor outcome and reduced disease-free survival ($p=<0.02$). Analysis of a large cohort is underway, and preliminary analysis of this independent set of samples also indicates that patients with primary tumors positive for Stefin A have an increased risk of recurrence ($P=<0.01$).

- Stefin A is not expressed in metastatic cells in mono-culture and is only induced in the same cells in the context of the tumour microenvironment. It can be induced in co-culture with appropriate stromal cells and extracellular matrix, but only to modest levels that do not mimic that seen in culture.

- Over-expression of Stefin A in tumor cells leads to a reduction in metastasis to lung and bone *in vivo*. This allows the hypothesis to be formed that Stefin A is a marker of the increased activity of cysteine proteases and that tipping the balance of protease:inhibitor interferes with metastatic progression. The interplay between cathepsins and their inhibitors may be important in breast cancer metastasis.

- Cathepsin B activity increases in highly metastatic primary tumors and Cathepsin B co-localises with Stefin A in primary tumors and lung and bone metastases in both the murine model and in human tissues.

- Findings derived from this award will form the basis of future grants aimed at determining the specific cathepsins that are key players in breast cancer metastasis to lung and bone.

REPORTABLE OUTCOMES

Awards

Finalist- Cure Cancer Australia Young Researcher of the Year (one of three finalists)

Travel Award of the 11th International Congress of Metastasis Research Society (MRS)

Publications

Chia, J., Anderson, R., Kusuma, N., **Parker, B**., Bidwell, B., Zamurs, L., Nice, E., Pouliot, N. Evidence for a role of laminin-10 in the metastatic progression of breast tumors. *Am. J. Pathol.*, Submitted.

Parker, B.S., Bidwell, B.N., Slavin, J., Pouliot, N., Henderson, M., and Anderson, R.L. Enhanced expression of the cathepsin inhibitor Stefin A is associated with metastatic progression in breast cancer. *Clinical Cancer Research*, submitted.

Wu, X., Chen, H., **Parker, B.S**., Rubin, E., Zhu, T., Lee, J.S., Argani, P. and Sukumar, S. (2006). HOXB7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial mesenchymal transition. *Cancer Research*, accepted with minor revision.

Eckhardt, B.L., **Parker, B.S**., van Laar, R.K., Restall, C.M., Natoli, A.L., Tavaria, M.D., Stanley, K.L., Sloan, E.K., Moseley, J.M., and Robin L. Anderson. (2005). Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix. *Molecular Cancer Research*, 3, 1-13.

Conference Presentations

Enhanced expression of the cathepsin inhibitor Stefin A is associated with metastasis in breast cancer. Parker, B.S, Bidwell, B.N, Slavin, J.L, Pouliot, N., Henderson, M. and Anderson, R.L. 11th International Congress of Metastasis Research Society, September 2006. Tokishima, Japan. Platform presentation.

Interplay between cathepsins and their inhibitor Stefin A regulates breast cancer metastasis to lung and bone. Parker, B.S., Bidwell, B.N., Pouliot, N.and Anderson, R.L. 18th Lorne Cancer Conference, February 2006. Lorne, Victoria, Australia.

Genetic aleteration in tumor epithelium and host endothelium associated with metastatic progression in a murine model of breast cancer metastasis. Parker, B.S and Anderson, R.L. 4th Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), June 2005. Philadelphia, Pennsylvania. Poster and platform presentation

CONCLUSIONS

Considerable effort is aimed towards a determination of the molecular pathways that contribute to breast cancer metastasis. The lack of clinically relevant models of spontaneous metastasis to bone hinder these studies. In this study, we have used a validated murine model of spontaneous metastasis to identify gene candidates that contribute to metastatic progression. Our study specifically investigates the expression of one of these genes, Stefin A, throughout metastatic progression in the mouse model and in human breast cancer.

We have identified that the cysteine cathepsin inhibitor Stefin A is enhanced throughout metastatic progression. Our studies have confirmed that Stefin A is induced in metastatic primary tumors and is further enhanced in matched metastases to lung and bone, in both murine and human tumors. In fact, lack of Stefin A expression decreased risk of recurrence and improved patient outcome in a small cohort study. Co-localisation studies have shown that Stefin A co-localizes with cathepsin B in primary tumors and metastases in lung and bone and we have also detected enhanced activity of cathepsin B in highly metastatic tumors in our murine model. Our data implicate the cysteine cathepsins and their endogenous inhibitor Stefin A in breast cancer progression and form the basis of future studies into the prognostic value of Stefin A and the inhibition of cathepsins as a target for therapy of metastatic breast cancer.

This work has many implications to breast cancer research. The use of a clinically relevant model of breast cancer metastasis was not only useful for finding gene candidates but is also of enormous importance in determining the functional role of such genes in the metastasis process. This has not been possible in other studies, and may be responsible for the lack of molecular markers as prognostic indicators and targets for treatment. The fact that we have verified expression of Stefin A in human breast cancer, with potential prognostic significance, reveals the clinical relevance of this model in investigating breast cancer metastasis.

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APPENDICES

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EDUCATION AND POSTDOCTORAL EXPERIENCE

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Field of Study: Breast Cancer
PI: Prof. Sara Sukumar

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Doctorate of Philosophy in Biochemistry **1998-2001**
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Supervisor: Prof. Don Phillips
Awarded First-Class Honours (H1)

La Trobe Univerisity
Bachelor of Science (Biological) **1997**
Majors: Biochemistry, Human Genetics

AWARDS/FELLOWSHIPS

- Travel Award of the 11th International Congress of Metastasis Research Society (MRS) **2006**
- US Army Department of Defense (DOD) Breast Cancer Research Program (BCRP) Concept Award **2005**
- Finalist- Cure Cancer Australia Young Researcher of the Year **2005**
- AACR Special Conference Scholar-in-Training Award (provided by the Avon Foundation) **2003**
- Awarded - Department of Defense Breast Cancer Research Program (BCRP) Postdoctoral Fellowship (July 2003-July 2006) **2003**
  - Susan Komen Foundation Postdoctoral Fellowship (declined)
  - Peter Doherty Postdoctoral Fellowship (March 2003-July 2003 then declined)
- Australian Society of Biochemistry and Molecular Biology (ASBMB) Travel Fellowship **2000**
- La Trobe University Postgraduate Scholarship (1998-2001) **1998**

MEMBERSHIPS/COMMITTEES

- Metastasis Research Society (MRS) member
- Australian Society of Medical Research (ASMR) member
- Australian Society of Biochemistry and Molecular Biology (ASBMB) member
- Peter MacCallum Postdoctoral Committee
- Peter MacCallum Infrastructure Committee
- Peter MacCallum Microarray Committee
- Peter MacCallum Tumour Biology Student Committee

SCIENTIFIC PUBLICATIONS

Chia, J., Anderson, R., Kusuma, N., **Parker, B**., Bidwell, B., Zamurs, L., Nice, E., Pouliot, N. Evidence for a role of laminin-10 in the metastatic progression of breast tumors. *Am. J. Pathol.,* Submitted.

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ONGOING RESEARCH SUPPORT

- DOD/BCRP- Postdoctoral Fellowship (DAMD17-03-1-0473)
  Title: Stromal gene expression in primary breast tumours that metastasize to bone
  July 2003-July 2006
  Role: PI
- DOD/BCRP- Concept Award (W81XWH-05-1-0444)
  Title: The role of Stefin A in breast cancer metastasis
  July 2005-July 2006
  Role: PI

CONFERENCE PRESENTATIONS

Interplay between cathepsins and their inhibitor Stefin A regulates breast cancer metastasis to lung and bone. Parker, B.S., Bidwell, B.N., Pouliot, N.and Anderson, R.L. 18th Lorne Cancer Conference, February 2006. Lorne, Victoria, Australia.

Genetic aleteration in tumor epithelium and host endothelium associated with metastatic progression in a murine model of breast cancer metastasis. Parker, B.S and Anderson, R.L. 4th Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), June 2005. Philadelphia, Pennsylvania. Poster **and** platform presentation

Cell specific gene expression profiling in a murine model of breast cancer metastasis. Parker, B.S. and Anderson, R.L. 10th International Congress of the Metastasis Research Society, September 2004. Genoa, Italy.

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